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Breast cancer is the second leading cause of cancer related death for women in the United States. Interestingly, breast cancer cells often highly express the Fas (CD95/APO-1) receptor, which is well established as an activator of apoptosis (programmed cell death) upon ligand binding. Fas induces apoptosis by recruiting proteins that form the death inducing signaling complex (DISC). Type I cells form large amounts of the DISC and internalize Fas, whereas in Type II cells Fas does not internalize and the DISC is almost undetectable. Additionally the Fas receptor has recently been shown to activate the nonapoptotic NF-κB and MAP kinase pathways upon receptor stimulation in either Type I or Type II cells. We can now demonstrate that in Type I cells the recruitment of DISC largely occurs after the receptor has moved into an endosomal compartment and blocking internalization prevents formation of the DISC. Receptor internalization is not required for NF-κB and Erk1/2 activation. Consequently dimerization of Fas complexes does not induce internalization of Fas nor apoptosis but is sufficient to induce nonapoptotic-signaling pathways and increases motility and invasiveness of tumor cells. Furthermore, we can demonstrate SNARK's role as a nonapoptotic kinase and promoter of motility and invasion.

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Table of Contents

PREFACE

Per the requirements of Department of Defense Breast Cancer Research Fellowship DAMD17-03-1-0200 I am submitting the annual update of research preformed as detailed in the updated SOW. It bears noting that the award was officially transferred to me on October 27, 2004. The original recipient, Bryan Barnhart, completed the requirements for his Ph.D and graduation from The University of Chicago and thus was not able to complete the experimental tasks detailed in his award. I applied for a transfer of the award on July 19, 2004 and submitted a revised statement of work on and subsequently received approval for the transfer of the award on October 27, 2004. Therefore I have only worked on the items listed in the updated SOW for less than a year. I understand the Bryan Barnhart did submit documentation to the command at the conclusion of his tenure as a graduate student. My financial administrator has advised me that we anticipate requesting an internal no cost extension to this award due to the late start of paying my salary from this award.

INTRODUCTION

Breast cancer cells often express the Fas (Fas/APO-1) receptor, which is well established as an activator of apoptosis upon ligand binding. Fas induces apoptosis by recruiting FADD and initiator caspases 8 and 10 forming a death inducing signaling complex (DISC)[1]. Type I cells form large amounts of the DISC and internalize Fas whereas in Type II cells Fas does not internalize and the DISC is almost undetectable [2-4]. Receptor internalization has been shown to limit extracellular signal stimulation and receptor recycling [2-6]. However, TNF-R1, a death receptor family member, has been shown to internalize upon binding of it ligand [7], TNFα, and TNF-R1 DISC formation requires internalization of the receptor [8]. Activation of the Fas receptor by upregulating the Fas ligand (FasL) is thought to be a desired response to chemotherapy and known to occur in various chemotherapy treatments. However, we and others have observed that Fas receptor stimulation also leads to activation of the nonapoptotic NF-κB and MAP kinase pathways which have known tumorigenic activities [9, 10]. Interestingly, TNF-R1 stimulation leads to activation of nonapoptotic signaling pathways at the plasma membrane and does not require internalization [11]. Both Type I and II cells activate the nonapoptotic pathways following FasL binding [10]. In cases where the receptor has a mutation in the death domain (DD) in only one allele apoptosis induction is blocked, but the nonapoptotic pathways are fully functional [12]. Fas stimulation of the breast cancer cell line MCF7-FB induces up-regulation of a defined number of mostly anti-apoptotic genes, resulting in increased motility and invasiveness of tumor cells [10]. Furthermore, the majority of these genes are known NF-κB target genes. We identified one of the Fas-regulated genes as the serine/threonine kinase (SNF1/AMP kinase related kinase (SNARK)), a member of the AMP kinase family, which is induced in response to various forms of metabolic stress. The mechanism of activation of the nonapoptotic pathways at the Fas receptor level is a primary tenant of this research proposal as described in task 1 and 2 of the SOW. We can now demonstrate that DISC formation requires internalization of Fas in Type I cells, and blocking internalization prevents formation of the DISC and thus provides an explanation for why the DISC is undetectable in Type II cells. In contrast activation of nonapoptotic pathways through Fas, such as NF-κB and Erk1/2, do not require receptor internalization. Furthermore, receptor dimerization is sufficient to activate the nonapoptotic pathways and to increase motility and invasiveness of MCF7-FB cells, but incapable of causing internalization and activation of the apoptotic response. Additionally, we show that over expression of SNARK renders tumor cells more resistant, whereas a kinase-inactive mutant of SNARK sensitizes cells to Fas-mediated apoptosis. Furthermore, small interfering RNA-mediated knockdown of SNARK increased the sensitivity of tumor cells to FasL- and TRAIL-induced apoptosis. Importantly, cells with reduced expression of SNARK also showed reduced motility and invasiveness in response to Fas engagement. SNARK therefore represents an NF-κB-regulated anti-apoptotic gene that contributes to the tumor-promoting activity of Fas in apoptosis-resistant tumor cells. The following details work complete and in progress that directly relates to the SOW.

RESEARCH

Task 1. Determine the mechanism of activation of NF-kB and MAP kinases by Fas at the receptor level

My original approach to this problem was to stimulate the Fas receptor with monoclonal antibody fragments. While this method is something that will be completed in the very near future to address the properties of monomeric binding of anti-Fas antibodies, we were able to take advantage of current commercially available antibodies against Fas that do not form higher aggregates and tested the activity of the stimulated dimer resulting in interesting data. We also employed a newly described techniques to isolate stimulated Fas receptor complexes. The information gained was such that we have recently submitted the work for publication. .

In Type I cells the DISC forms following receptor internalization: We first wanted to determine the order in which the DISC forms with respect to the internalization events. We took advantage of a novel method used to identify the requirement for internalization of TNF-R1 in the formation of the DISC [8]. We selected cells lines from a group or 22 tumor cells lines recently classified as Type I or Type II - Type I cells form large amounts of the DISC, whereas Type II cells do not [2]. Our choice of solid tumor cells lines HCT15 (Type II) and ACHN (Type I) is based on their relative levels of Fas expression. ACHN cells express slightly less Fas than HCT15. ACHN (Figure 1A) and HCT15 (Figure 1B) cells were incubated with biotinylated anti-APO-1 mAb. The labeled antibody was isolated as previously described at different time points post stimulation. Western blotting for marker proteins typical of endosomal/lysosomal vesicles was used to detect the molecules associated with the isolates. This method allowed us to follow the labeled receptor through the endosomal/lysosomal pathway. Maximum detection of Rab4, the marker for endosomal trafficking, occurred at 5 min in ACHN cells, marking the time point at which the receptor began to internalize (Figure 1A, lanes 1-5). The early endosomal marker protein, endosomal autoantigen 1 (EEA1), appeared at 5 min and peaked at 30 min indicating that the vesicles containing the activated Fas had moved inside the cell and arrived in an endosomal compartment. The lysosomal marker cathepsin D began to appear at 5 min and kept increasing until 60 min indicating that Fas and its associated proteins moved into lysosomes as early as 5 min after stimulation (Figure 1A, lanes 1-5, top panels). In contrast to the Type II cell line HCT15 where all three marker proteins increased only slightly 60 min after stimulation indicating a lack of significant internalization by activated Fas. We then analyzed the isolates for the presence of recruited DISC components. In ACHN cells

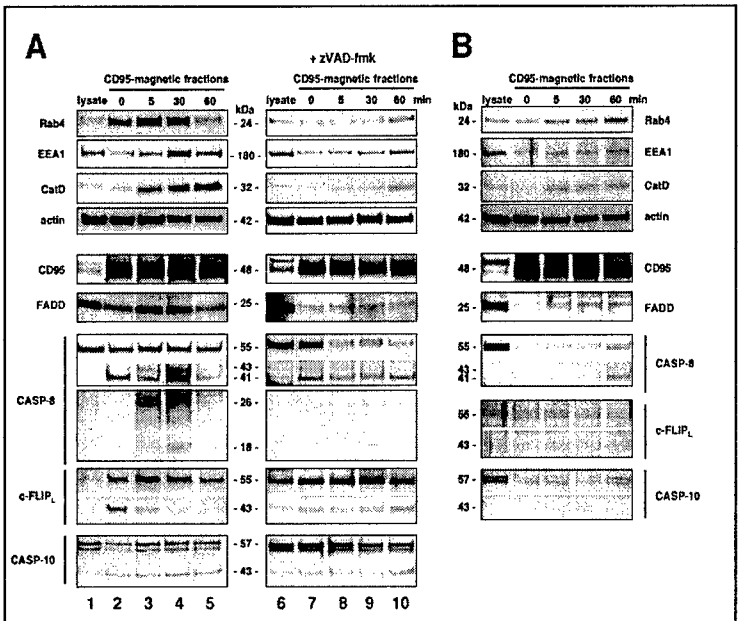

Fig 1. Internalization and endosomal maturation of Fas receptors and recruitment of FADD, caspase-8, cFLIPL and caspase-10 to Fas magnetic fractions isolated from ACHN and HCT15 cells. (**A**) Time course of intracellular Fas-receptosome trafficking in ACHN cells and effect of zVAD-fmk. Total cell lysates or magnetic fractions derived after 0, 5, 30 and 60 min of anti-APO-1 mAb treatment in the absence or presence of zVAD-fmk were analyzed for signature proteins of endosomal maturation (Rab4, EEA1) and lysosomes (32 kDa CatD), actin, Fas, and the DISC composed of FADD, caspase- 8, c-FLIPL and caspase-10 by Western blotting. (**B**) Time course of recruitment of endosomal, lysosomal and the DISC proteins to Fas magnetic fractions isolated from HCT15 cells.

recruitment of FADD, caspase-8, caspase-10 and activation of caspase-8 peaked at 30 min, a time point at which most of the receptor had moved into an EEA1 containing compartment. Only c-$FLIP_L$ was found to be recruited and cleaved earlier. The data demonstrate that the major recruitment of the DISC components occurs after the onset of internalization of Fas in ACHN cells, and no significant recruitment of DISC components to activated Fas was detected in HCT15 cells during the course of the experiment consistent with the inability of these cells to internalize Fas (Figure 1B).

It has been shown that internalization of Fas was block in Type I cells by inhibiting the activity of caspase-8 [13, 14]. Therefore inhibiting the activity of caspase-8 should prevent the formation of the DISC. Repeating the experiment with ACHN cells in the presence of zVAD-fmk, a caspase inhibitor (Figure 1A, right panel), we observed that internalization of Fas was blocked in these cells and there was no recruitment of the DISC components. The observation is such that zVAD-fmk treated ACHN cells behaved much like the HCT15 cells with only a very late and minor recruitment of DISC components to the activated receptor. Our data suggest that activated Fas requires internalization for the recruitment of most of the apoptotic signaling molecules in Type I cells. When internalization is blocked none of the DISC components are efficiently recruited by Fas, a situation very similar to that found in Type II cells which do not internalize Fas.

IgG2b anti-APO-1 does not induce clustering, internalization, DISC formation, or apoptosis: DISC formation in Type I cells requires the internalization of Fas to fully activate the apoptotic pathway. Type I and Type II cells also activate nonapoptotic pathways, NF-κB and MAPK, upon Fas stimulation. This is true for many types of tumor cells including breast cancer cells [10]. Type II cells do not internalize Fas and it has been previously observed that NF-κB is activated upon Fas stimulation under conditions that inhibit receptor internalization [2]. Furthermore, monoallelic death domain mutations are though to block apoptotic signaling by preventing trimerization of the aggregate receptors [15]. We sought to determine whether dimeric activation of the receptor was insufficient to cause apoptosis. The anti-APO-1 IgG3 antibody is thought to cause receptor aggregation and subsequent activation of apoptosis via Fc-Fc interactions [16]. The IgG2a or b isotype has the same specificity for Fas but lacks the ability to promote Fc-Fc interactions. Therefore IgG2b binding to Fas will be limited to dimerization without aggregation. Consistent with previous data we found that the IgG2b antibody was insufficient to cause apoptosis of SKW6.4 and H9 cells until the antibody was crosslinked by protein A (Figure 2A). To test whether the nontoxic anti- APO-1 IgG2b isotype could induce clustering and internalization of Fas on Type I cells we added

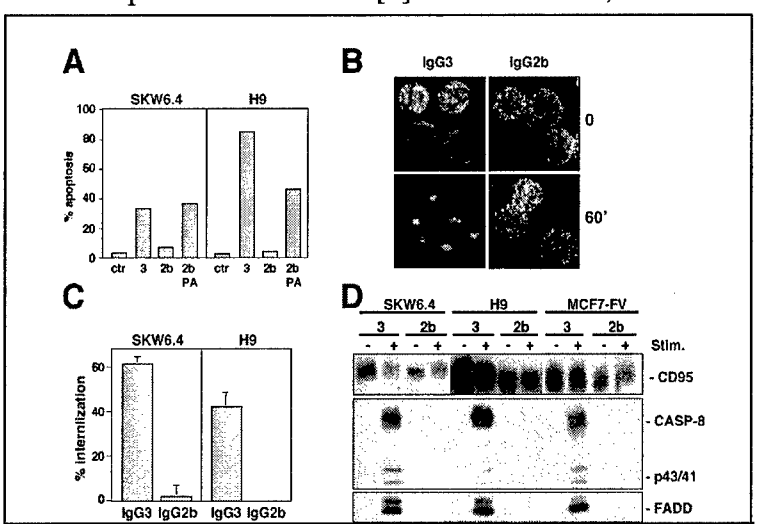

Fig 2. The IgG2b anti-APO-1 switch variant does not induce internalization or death inType I cells. (**A**) DNA fragmentation of cells treated with control IgG (ctr), anti-APO-1 IgG3 (3) or anti-APO-1 IgG2b (2b) without or with 100 ng/ml protein A (PA) added. The experiment was done in triplicates. Shown is the mean with standard deviation. (**B**) Clustering of Fas in SKW6.4 cells after stimulation with FITC-conjugated anti-APO-1 IgG2b or IgG3 for 1 hour. (**C**) Quantification of the number of cells with internalized Fas after 1 hr of stimulation with either IgG3 or IgG2b anti-APO-1. The experiment was done in triplicates. Shown is the mean with standard deviation. (**D**) Standard DISC analysis of SKW6.4, H9 and MCF7-FV cells stimulated with anti-APO-1 IgG3 or IgG2b.

FITC conjugated anti-APO-1 IgG2b to SKW6.4 and H9 cells. This antibody did not induce any detectable clustering of Fas (Figure 2B and data not shown), and no internalization could be detected in cells treated with the IgG2b antibody (Figure 2C). The IgG2b anti-APO-1 Ab did not induce formation of the DISC (Figure 2D), which is consistent with our previous data for the requirement of Fas internalization in the DISC formation. These data suggest that divalent antibody induced dimerization of preassociated Fas complexes is insufficient to trigger clustering or internalization of Fas, formation of the DISC or apoptosis through Fas.

IgG2b anti-APO-1 induces activation of NF-κB and MAPK: IgG2b binding is insufficient cause internalization and apoptosis. We therefore tested whether the antibody could activate Fas stimulated nonapoptotic pathway. MCF7-Fas Vector (MCF7-FV) cells which have been shown to form a DISC when stimulated with IgG3 anti-APO-1 and efficiently activate nonapoptotic pathways when stimulated through Fas, showed no clustering, internalization or formation of the DISC when stimulated with IgG2b anti-APO-1 (data not shown and Figure 2D). However this Ab induced activation of NF-kB and Erk1/2 in MCF7-FV cells as effectively as the IgG3 isotype in apoptosis resistant MCF7-FB cells (Figure 3A and B) suggesting that dimerization of Fas complexes at the cell surface is sufficient to activate nonapoptotic pathways.

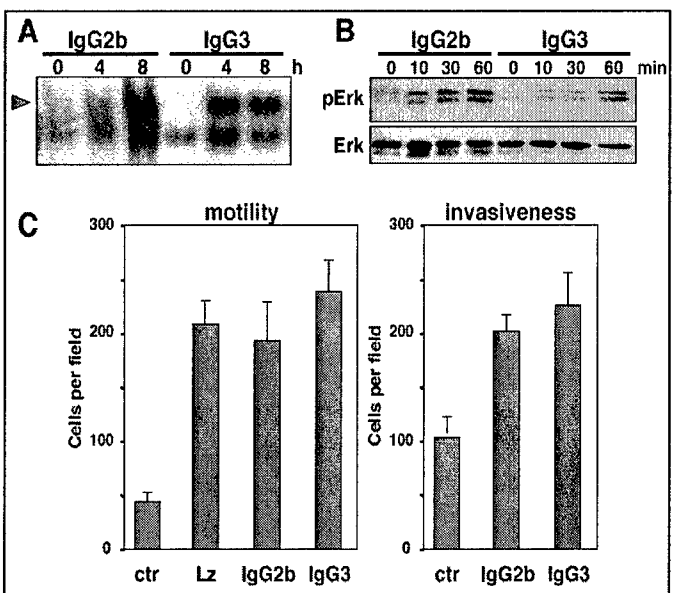

Fig 3. Dimerizationn of Fas complexes is sufficient to induce nonapoptotic pathways through Fas. (**A**) EMSA analysis of MCF7-FV cells stimulated with IgG2b anti-APO-1 and MCF7-FB cells stimulated with IgG3 anti-APO-1 for the indicated times. The arrowhead marks the migration of the p50/p65 NF-_B heterodimer. (**B**) MCF7-FV cells stimulated with IgG2b anti-APO-1 and MCF7-FB cells stimulated with IgG3 anti-APO-1 for the indicated times were subjected to Western blot analysis for phospho-Erk (pErk) and Erk. (**C**) MCF7-FB cells were treated with either control IgG (ctr), LzFasL (Lz), IgG2b or IgG3 anti-APO-1 were subjected to either a motility (left) or Invasiveness (right) assay.

IgG2b anti-APO-1 induces motility and invasiveness: Finally we tested whether stimulation of tumor cells with the IgG2b anti-APO-1 Ab would induce an increase in in vitro motility and invasiveness (Figure 3C). Both of these responses were observed when MCF7-FB cells were incubated with the IgG2b Ab. Our data suggest that signaling through Fas causes tumor cells to become more invasive when the signal originates from cell surface and does not require formation of the DISC or the internalizing receptor. Consistent with this conclusion is the observation that we did not find any difference in Fas induced activation of nonapoptotic pathways between Type I and Type II cells (data not shown).

Task 1 Outlook: I am currently generating the antibody $F(ab')_2$ fragments using the recently purchased ImmunoPure $F(ab')_2$ Preparation Kit from Pierce Biotechnologies. We predict that we will confirm our observation noted above for dimeric activation of the receptor. However, we will be unable to visually observe internalization with this method. Thus the previously described work is the foundation for this next step. I have also purchased the ImmunoPure Fab Preparation Kit for Pierce. I wll generate the Fab fragment and complete the observation to determine if the monomeric receptor is sufficient to activate the nonapoptotic pathways and stimulate motility and invasion.

Task 2. Test known Fas signaling molecules as mediators of activation of NF-kB and MAP kinases by Fas .

The issues relating to Task 2 will be addressed during the next 12 month period and the approach detailed in the SOW will be completed. Furthermore, I have begun planning a wide scale approach to the problem in an attempt to identify the molecular mediators of activation of the nonapoptotic pathways unique to Fas stimulation, I will employ the use of an siRNA library to screen for molecules that selectively upregulate NF-κB in Fas when compared to TNFα stimulation. We have previously observed that NF-κB upregulation in TNFα stimulation is mediated through RIP and that RIP deficient cells can upregulate NF-κB upon Fas stimulation but not upon TNFα stimulation (data not shown). This allows us to search for a potentially unique mediator that regulates NF-κB in Fas stimulated cells. Our screen will allow us to identify NF-κB activity in a Fas dependant nature. We will employ an NF-κB driven GFP reporter to monitor the activity of NF-κB in transfected A549 cells. Through selection under screening using the Lenti viral knock-down library we will select for cells that have a high GFP expression upon TNFα stimulation but no GFP expression upon Fas stimulation. The use of the library allows for rapid detection and identification of the required mediators of Fas directed NF-κB activity.

Task 3. Determine the importance for SNARK for Fas induced increase of invasiveness in breast cancer cells.

Work on task 3 had begun during the transfer period of the award. We have since published the results pertaining to SNARK. Editorial requirements for revisions of the original submission demanded that we address issues directly relating to this task while the transfer of the award was under consideration and awaiting final approval. The approval for publication of this work and the approval for the transfer of the award occurred at relatively the same time. I will detail the results of the work that I completed as they pertain to the fulfillment of task 3. Parts of task 3a and all of task

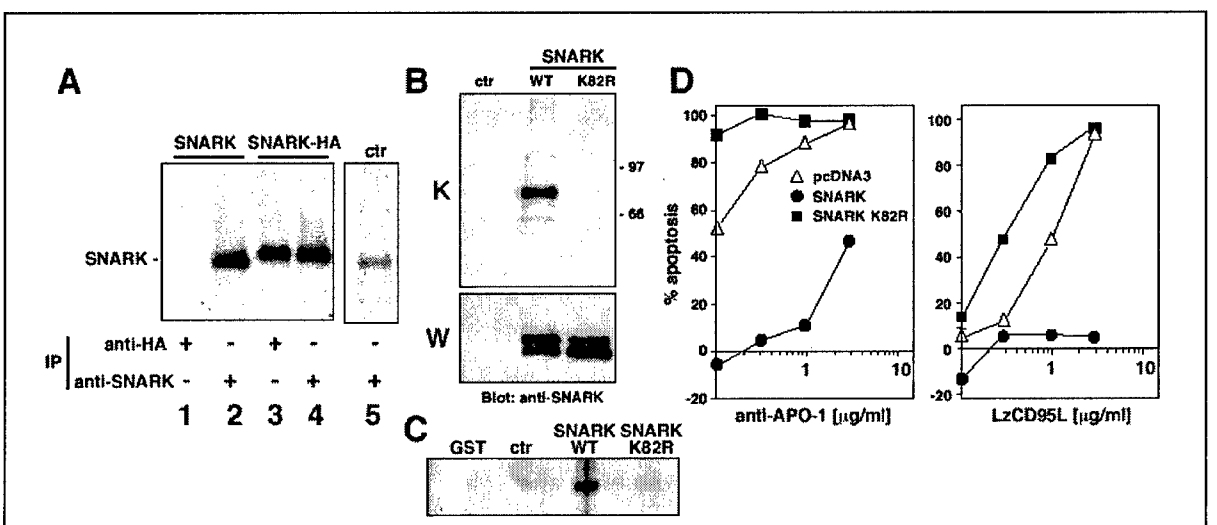

Fig 4. SNARK inhibits Fas induced apoptosis. *A, In vitro* kinase assay of SNARK or HA-SNARK immunoprecipitated with the indicated antibodies from lysates of transiently transfected 293T cells. *B,* 293T cells were transfected with wild-type or K82R mutant SNARK and an autophosphorylation assay (K) was performed. Equal expression of SNARK and the mutant protein in 293T lysates was controlled by an anti-SNARK immuno-blot (W). *C, In vitro* kinase assay on the SAMS peptide. Lysates of empty vector (ctr), SNARK wild type or K82R SNARK transfected 293T cells were used to perform a kinase assay using GST-SAMS as substrate. The same amount of GST alone was incubated with the SNARK wild-type transfected 293T lysate and used as control to demonstrate the specific phosphorylation of SAMS peptide. *D,* HuSNARK inhibits Fas-induced apoptosis and kinase-dead SNARK (SNARK K82R) sensitizes ACHN cells to Fas induced apoptosis. ACHN cells were transfected with the indicated constructs and stimulated 16 hours with the indicated stimuli. The quantification of cell death was performed with a MTS assay in triplicates.

3b have been completed. I have scheduled to begin work on the uncompleted issues describe in task 3 for later in the year.

Kinase Inactive SNARK Sensitizes Cells to Fas Mediated Apoptosis - We overexpressed the cloned human SNARK kinase in 293T cells and determined that it is an active kinase with typical autophosphorylation properties and with an apparent size of 70 kDa (Fig. 4A). We also detected a phosphorylated protein in control transfected cells that comigrated with immunoprecipitated untagged SNARK likely corresponding to active endogenously expressed SNARK (Fig. 4A, lane 5). To determine the function of endogenous SNARK in Fas induced apoptosis we generated a kinase inactive mutant (Fig. 4B). This mutant was generated by replacing lysine 82, a highly conserved position in the AMP kinase family, with arginine. This residue change has been shown to act in a dominant negative fashion when overexpressed. The kinase function of the K82R mutant of SNARK was inactive as it did not undergo autophosphorylation (Fig. 4B) or phosphorylate a substrate comprising a fusion protein of GST with the SAMS peptide (Fig. 4C), the optimal sequence for phosphorylation by members of the AMP kinase family [17]. Overexpression of either SNARK or SNARK K82R in the Type I cell line ACHN and stimulation of Fas resulted in the cells being significantly protected from apoptosis by the expression of SNARK (Fig 4D). This inhibition was dependent on the kinase activity of SNARK since SNARK K82R could not protect cells from Fas mediated apoptosis. In contrast, SNARK K82R rendered cells even more susceptible to Fas mediated apoptosis suggesting that endogenous SNARK was dominant negatively inhibited by the overexpression of mutant SNARK. Transient overexpression of SNARK also protected another Type I cell lines CAKI-1 from both Fas ligand (FasL) and TRAIL induced apoptosis (data not shown).

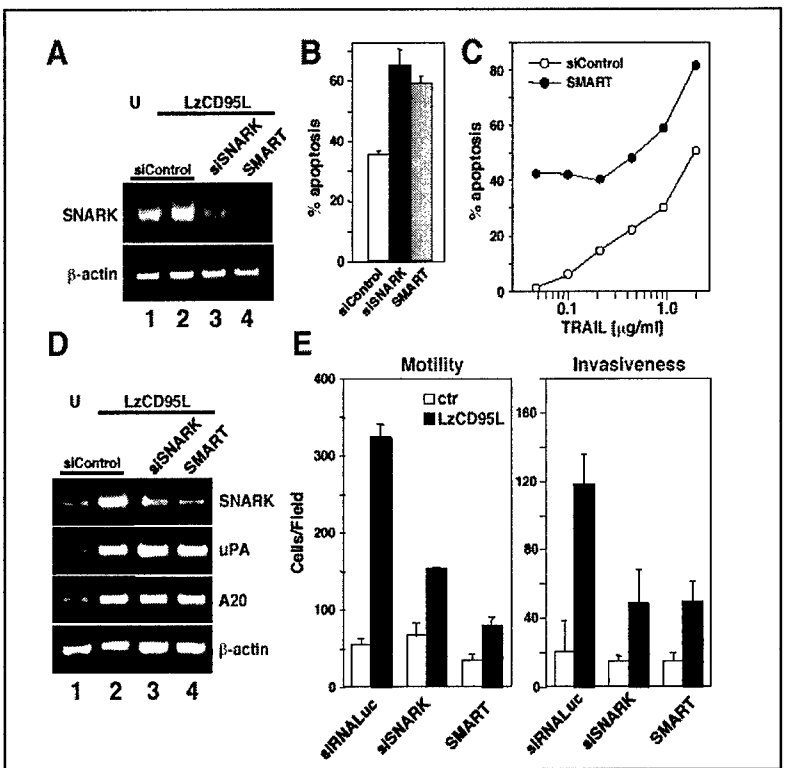

Fig 5. SNARK knock-down increases the sensitivity toward FasL and TRAILtriggered apoptosis and inhibits Fas-induced motility and invasiveness. *A,* Semiquantitative RT-PCR of ACHN cells transfected with a siRNA control (siRNA Luciferase-Cy3) or with two different SNARK specific siRNAs for 24 hours (see experimental procedures), left untreated (U) treated or Treated for two hours with LzFasL. Cells were pre-incubated 1 hour with 40 μM of zVAD-fmk. *B, C,* ACHN cells were transfected with siRNA control or siRNA SNARK and after 24 hours incubated for 16 hours with 1 μg/ml of LzFasL (B) or the indicated concentrations of TRAIL (C) and cell death was quantified by a MTS assay. *D,* Semi-quantitative RT-PCR of MCF7-FB cells transfected with a siRNAs and treated with LzFasL as in A. *E,* The MCF7-FB cells were transfected with the indicated siRNA constructs and after 24 hours were subjected to *in vitro* motility and invasiveness assays.

SNARK Is an Antiapoptotic Gene Required for Fas-induced Increase of Motility and Invasiveness of Fas Mediated Apoptosis Resistant Tumor Cells - To directly determine the function of SNARK as a Fas regulated gene we reduced the expression of SNARK using siRNAs. RT-PCR of ACHN cells found endogenous expression of SNARK (Fig. 5A, lane 1); however expression could be enhanced by treating cells with LzFasL (Fig. 5, lane 2). Treating the cells with two

independent siRNAs (siSNARK and an siRNA SMART pool) for 48 hrs resulted in a significant reduction of SNARK mRNA (Fig. 5A, lanes 3 and 4). ACHN cells showed increase sensitivity to Fas (Fig. 4B) or TRAIL (Fig. 5C) mediated apoptosis when treated with the SNARK siRNA. These experiments confirmed the antiapoptotic activity of endogenous SNARK for death receptor induced apoptosis. SNARK was one of only 17 Fas induced genes in MCF7-FB cells, which respond to Fas triggering with increased motility and invasiveness [10]. We therefore tested whether the loss of SNARK would inhibit the Fas mediated migration and invasiveness characteristic of these cells. As reported previously MCF7-FB cells responded to stimulation with LzFasL with upregulation of typical NF-κB target genes such as urokinase plasminogen activator (uPA), A20 and of SNARK (Fig. 5D, lanes 1 and 2). Treating the cells with either of the siRNAs directed at SNARK mRNA resulted in a significantly reduced upregulation of SNARK without affecting induction of uPA or A20 (Fig. 5D, lanes 3 and 4). We then subjected siRNA SNARK-treated MCF7-FB cells to motility and invasiveness assays. Both Fas induced motility and invasiveness were severely reduced in siSNARK treated cells without affecting the general viability of these cells (data not shown), identifying SNARK as a gene that regulates this novel nonapoptotic activity of Fas.

Both AMPK and another member of the AMPK family, ARK5, which shows 55% overall homology with SNARK, including 84% identity within the N-terminal kinase domain, were recently described as having antiapoptotic activity [18, 19]. Overexpression of ARK5 rendered cells more resistant to TNFα, TRAIL, and glucose deprivation. It was therefore postulated that ARK5 could promote tumor cell survival during nutrient starvation. The recent recognition that Fas has a tumorigenic activity on tumor cells both in vitro [10] as well as in vivo [20] suggests the existence of protumorigenic genes that are induced in response to Fas stimulation. SNARK induction requires activation of NF-κB, which was shown to be critical for the Fas protumorigenic activities.

Task 3 Outlook: I am working on the generation of stably transfected SNARK and dominant negative (dn) SNARK in various cell lines. Once we have established this I will repeat the transfections in murine cells. I have already completed RT-PCR of murine lung cancer cell line 3LL and confirmed the presence of the murine ortholog of SNARK. Preceding the in-vivo observations of the effect of SNARK and dn-SNARK in mice, I will characterize the transfected cells lines to see if the previous observations in human cells are recapitulated in the murine system.

CONCLUSIONS

The research focus of this reporting period has largely followed the time frame delineated in the updated SOW. The minor deviations from the original outline provided us with valuable information pertaining the mechanisms of Fas mediated signaling. In summary, Type I cells require internalization of Fas for formation of the DISC and efficient induction of apoptosis. In contrast to Type II cells, which do not require internalization to induce apoptosis. Activation of nonapoptotic signaling pathways by Fas generally does not require receptor internalization regardless of whether cells are Type I or Type II and dimerization of receptor complexes is sufficient to induce activation of NF-κB and MAP kinases. These data suggest that signaling pathways emanating from Fas are initiated by signaling complexes that differ not only quantitatively but also in their subcellular localization. Nonapoptotic signaling pathways can be activated through nonaggregated receptors at the plasma membrane. This is in contrast to the apoptotic signaling observed in Type I cells which requires aggregation of Fas and movement of the activated receptor into plasma membrane derived endosomes to recruit sufficient amounts of FADD and caspase-8 needed for cell death. This novel pathway to activate caspase-8 may be important for targeting the caspase to its intracellular substrates. Additionally, our lab recently demonstrated that uPA, one of the NF-κB regulated genes induced by Fas, is critical for the Fas induced motility and invasiveness of apoptosis resistant breast cancer cells cells. Blocking the activity of uPA blocked the ability of cells to invade in response to

Fas stimulation. SNARK is the second of the recently identified Fas induced genes tested and siRNA induced down-modulation of SNARK demonstrated SNARK's importance for the novel Fas dependent tumorigenic activities. Reducing the expression of SNARK did not affect the expression of uPA suggesting that SNARK is not required for the induction of uPA. The fact that Fas activates at least 5 different nonapoptotic signaling pathways independently resulting in induction of multiple genes suggests that the regulation of these activities is complex. The outlook for future research in Fas mediated tumorigenisis has enormous potential and has evolved into my doctoral thesis proposal. This unique and diverse signaling process has many questions that demand to be addressed. The answers yet to be found may prove to be extremely beneficial not only to women suffering from breast cancer but to all people who suffer from cancer.

REPORTABLE OUTCOMES

Manuscript:

*Feig, C., *Tchikov, V., *Schickel, R., Vijayan, S., Schuetze, S and Peter, M.E. The role of receptor internalization in CD95 signaling. submitted.* shared first authorship

Publications:

Legembre, P., Schickel, R. Barnhart, B.C. and Peter, M.E. (2004) Identification of SNF1/AMPK-related kinase as a NF-kB regulated antiapoptotic kinase involved in CD95-induced motility and invasiveness. J Biol Chem, 279, 46742-46747.

Presentations:

The University of Chicago Committee on Cancer Biology, Works in Progress, April 2005

References

1. Peter, M.E. and P.H. Krammer, *The CD95(APO-1/Fas) DISC and beyond.* Cell Death Differ, 2003. **10**(1): p. 26-35.
2. Algeciras-Schimnich, A. and M.E. Peter, *Actin dependent CD95 internalization is specific for Type I cells.* FEBS Lett, 2003. **546**(2-3): p. 185-8.
3. Algeciras-Schimnich, A., et al., *Two CD95 tumor classes with different sensitivities to antitumor drugs.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11445-50.
4. Barnhart, B.C., E.C. Alappat, and M.E. Peter, *The CD95 type I/type II model.* Semin Immunol, 2003. **15**(3): p. 185-93.
5. Ferguson, S.S., *Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling.* Pharmacol Rev, 2001. **53**(1): p. 1-24.
6. Trowbridge, I.S., *Endocytosis and signals for internalization.* Curr Opin Cell Biol, 1991. **3**(4): p. 634-41.
7. Schutze, S., et al., *Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling.* J Biol Chem, 1999. **274**(15): p. 10203-12.
8. Schneider-Brachert, W., et al., *Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles.* Immunity, 2004. **21**(3): p. 415-28.
9. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Non-apoptotic Fas signaling.* Cytokine Growth Factor Rev, 2003. **14**(1): p. 53-66.
10. Barnhart, B.C., et al., *CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells.* Embo J, 2004. **23**(15): p. 3175-85.
11. Micheau, O. and J. Tschopp, *Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes.* Cell, 2003. **114**(2): p. 181-90.
12. Legembre, P., et al., *Induction of apoptosis and activation of NF-kappaB by CD95 require different signalling thresholds.* EMBO Rep, 2004. **5**(11): p. 1084-9.
13. Algeciras-Schimnich, A., et al., *Molecular ordering of the initial signaling events of CD95.* Mol Cell Biol, 2002. **22**(1): p. 207-20.

14. Stegh, A.H., et al., *Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein.* J Biol Chem, 2002. **277**(6): p. 4351-60.
15. Martin, D.A., et al., *Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4552-7.
16. Dhein, J., et al., *Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens.* J Immunol, 1992. **149**(10): p. 3166-73.
17. Sullivan, J.E., et al., *Characterisation of 5'-AMP-activated protein kinase in human liver using specific peptide substrates and the effects of 5'-AMP analogues on enzyme activity.* Biochem Biophys Res Commun, 1994. **200**(3): p. 1551-6.
18. Suzuki, A., et al., *Induction of cell-cell detachment during glucose starvation through F-actin conversion by SNARK, the fourth member of the AMP-activated protein kinase catalytic subunit family.* Biochem Biophys Res Commun, 2003. **311**(1): p. 156-61.
19. Blazquez, C., et al., *The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes.* FEBS Lett, 2001. **489**(2-3): p. 149-53.
20. Lee, J.K., et al., *Lack of FasL-mediated killing leads to in vivo tumor promotion in mouse Lewis lung cancer.* Apoptosis, 2003. **8**(2): p. 151-60.